Environmental Health Perspectives
Vol. 62, pp. 105-108, 1985

Detection of 8,9-Dihydro-(7′-guanyl)-9-hydroxyaflatoxin B_1 in Human Urine

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A possible role of aflatoxin B_1 (AFB) in the etiology of human liver cancer has been suggested from several epidemiological studies. This has been based upon the association between consumption of AFB-contaminated food and the liver cancer incidence in different parts of the world. To further establish the role of AFB as a major factor, we initiated a pilot study in three different districts of Kenya to determine the number of individuals exposed to significant amounts of AFB as measured by the urinary excretion of 8,9-dihydro-8-(7-guanyl)-9-hydroxyaflatoxin B_1 (AFB-Gua), an adduct formed between the ultimate carcinogenic form of AFB and nucleic acids. This product has previously been detected in urine from rats treated with AFB. Urine collected at the outpatient clinics at the district hospitals were concentrated on C_{18} Sep-Pak columns and analyzed by high-pressure liquid chromatography under two different chromatographic conditions. The chemical identity of the samples showing a positive response in both chromatographic systems was verified by synchronous scanning fluorescence spectrophotometry.

The highest number of individuals with detectable urinary AFB-Gua lived in either Murang'a district or the neighboring Meru and Embu districts. In Murang'a district a rate of 12% was observed in the January–March period, while only 1 of 32 patients (3%) had a detectable exposure in July–August. A significant lower number of urine samples were positive for AFB-Gua in the other areas. However, the collection of samples in these areas was during April–June, a period with lower AFB contamination of the food. A higher number of men were positive for AFB-Gua in the urine than women. The results indicate that a significant number of individuals in an area of high liver cancer risk has been exposed to AFB. However, the present technology is not sensitive enough to detect lower level of AFB exposure, and the method is too laborious to be used for a large epidemiological study.

Background

Aflatoxin B_1, a mycotoxin produced by *Aspergillus flavus*, is a potent liver carcinogen in rodents and non-human primates (*1,2*). A number of epidemiological studies have been conducted in Asia and Africa which have shown a positive association between the dietary intake of AFB and the incidence of liver cancer (*3–5*). As the level of AFB was determined in either market samples or in prepared food ready for consumption, it has been difficult to calculate an exact individual exposure level. An approach to determine the biological dose of the carcinogen would be to measure AFB and/or its metabolites in the body fluids, although it may not represent the genotoxic dose of AFB. Tsuboi et al. (*6*) have reported the presence of AFB in serum samples of male Japanese subjects as determined by radioimmunoassay and high-pressure liquid chromatography (HPLC). Specific AFB metabolites or total AFB metabolites have been detected in human urine samples by either chromatography or immunological methods (*7–9*). An accurate measure for the genotoxic dose would be to detect product(s) formed between the ultimate form of the carcinogen and the genetic material. This approach has successfully been demonstrated by detecting carcinogen–DNA adducts in the white blood cell DNA isolated from individuals exposed to high level of benzo(a)pyrene (*10,11*). In cells and tissues treated with carcinogens, the initial damage is quickly repaired by enzymatic processes. The fate of these products is relatively unexplored, but they may serve as good indicators of carcinogen exposure. 7-Methylguanine (*12*) and AFB-Gua (*13*) have been detected in rat urine after treatment of the animals with dimethylnitrosamine and AFB, respectively. These repair products are probably formed by spontaneous depurination rather than by an active DNA-repair process. The major AFB-DNA adduct *in vivo* and *in vitro* is formed by the interaction of AFB-8,9-oxide and the 7-position of guanine in DNA (*14*). This adduct accounts for more than 80% of the AFB-DNA adducts in rat liver 2 hr after administration of AFB (*15*). The same adduct was also detected in cultured fetal liver explants after incubation with AFB

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(*16*). Formation of AFB-Gua in DNA induces a strong positive charge in the imidazole ring, resulting in either depurination of AFB-Gua or an opening of the imidazole ring to form two structurally related adducts, one of which has been identified as 8,9-dihydro-9-(N5-formyl-2′,5′,6′-triamino-4′-oxo-N5-pyrimidyl)-9-hydroxy-AFB (*17,18*). The latter adduct appears to be fairly stable in the DNA, although an enzyme isolated from *E. coli* has been shown to excise this product (*19*). The amount of AFB-Gua in the urine after treating rats with AFB corresponded to approximately 1% of the administered dose (*13,20*), and a relationship between the dose of AFB-Gua has also been detected for up to 72 hr in the tissue culture media after treating a hepatocellular carcinoma cell line with AFB in the presence of liver microsomes. In a preliminary communication we have reported the presence of a putative AFB-Gua product in urine collected from individuals living in areas with suspected exposure to AFB (*21*).

Procedure and Results

Urine samples (minimum 25 mL) were collected at the outpatient clinic at Murang'a, Machakos, and Makueni District Hospitals and the Kenyatta National Hospital, Nairobi using sterile, disposable containers. These donors did not have a clinical history of liver disorders, but consulted the clinic for malaise. The age and sex distribution of the donors is given in Table 1. The samples were immediately put on ice and transferred to the laboratory for processing by a modification of the procedure by Bennett et al. (*13*). Samples were adjusted to pH 5 and made 7% in methanol followed by centrifugation at 4°C (1500 *g* for 10 min) to remove any particulates. The supernatant was submitted to initial clean-up on a C18 Sep-Pak cartridge (Waters Associates, Waltham, MA., USA) using a Sep-Pak cartridge rack and a maximum flow rate of 2mL/min. After washing with 5 mL 10% methanol and 5 mL 7% acetonitrile, the samples were shipped to the USA. The cartridges were kept at -70°C until analysis by high pressure chromatography. The cartridges were eluted with 80% methanol (10 mL) and concentrated to 0.5 mL by using a Speedivac evaporator and subjected to further clean-up by HPLC with the use of a C18 Bondapak column and 18% ethanol–10 mM ammonium formate (pH 5.1) as eluent with a flow rate of 1 mL/min. Under these conditions, AFB-Gua eluted at 21 min, and fractions from 20 to 23 min were collected. Several urinary compounds absorbing at 365 nm co-migrated with AFB-Gua on the C18 Bondapak column, but this peak was resolved into several components on an Ultrasil-Si (Altex) column using 4.5% acetonitrile (flow rate 1 mL/min). The eluent was monitored at 365 nm (Waters model 44 spectrophotometer). AFB-Gua eluted at 5.5 min on this column. The identity of the putative AFB-Gua adduct was verified by cochromatography with minute amounts of synthetically produced 3HAFB-Gua (*22*) once every day. The recovery of AFB-Gua by this procedure was approximately 75%. The chemical identity of the positive samples was verified by fluorescence spectrophotometry by use of synchronous luminescence and photon counting (*23*). The fluorescence was measured in a quartz cell (10 mm light path), and both the excitation and emission monochromators were set at 5 nm band width. The difference was 34 nm, the emission being 34 nm higher than that of excitation, and emission and excitation were scanned simultaneously (synchronous scanning). Under these conditions, both AFB-Gua isolated by HPLC from chemically modified DNA and the urine samples positive in both HPLC systems gave a characteristic fluorescence with a peak emission of 415 nm, when excited at 381 nm (*21*). When the samples were made alkaline no bathochromic shift in the spectrum was observed. Presently, a total of 355 urine samples has been analyzed for the presence of AFB-Gua. A sample was considered positive for AFB-Gua if it gave a UV absorption at 365 nm in both HPLC systems and had the characteristic synchronous fluorescent spectrum. The lower level of detectability was 0.3 pmole AFB-Gua.

A total of 24 samples (6.7%) were positive for AFB-Gua by these criteria (Table 2), with the highest frequency in patients from Murang'a district. A relatively high number of positive cases was also detected at the outpatient clinic at Kenyatta National Hospital, Nairobi. However, when these cases were analyzed according to the patients' home location (Table 3) only patients living in Murang'a, Kiambu, and Meru/Embu districts were positive.

It is difficult to compare the rate of AFB-Gua positive samples in the different areas, as the collection took

Table 1. Age and sex distribution.

	Male		Female	
Age group	Number of cases	% of cases positive for AFB-Gua	Number of cases	% of cases positive for AFB-Gua
11–20	38	7.9	37	10.8
21–30	36	8.3	73	6.8
31–40	24	12.5	36	8.3
41–50	12	0	25	0
51–60	17	11.8	19	0
>61	19	15.8	19	0
Total	146	9.0	209	5.5

Table 2. Analysis of urine samples for AFB-GuaI.

Collection site	No. of samples analyzed	Cases positive[a]
Kenyatta National Hospital, Nairobi		
January–March	128	11 (8.6%)
Murang'a District Hospital		
January–March	61	7 (11.5%)
July–September	32	1 (3.1%)
Machacos District Hospital		
April–June	15	1 (7%)
Makueni District Hospital		
April–June	119	4 (3%)
Total	355	24 (6.7%)

[a] Limit of detectability was 0.3 pmole.

part in different seasons. It has previously been shown that the consumption of AFB had a seasonal variation (*4*). An interesting seasonal variation in the number of positive cases was observed in Murang'a district: there was a positive case rate of 11.5% in January–March but only 3% in July–September. The sex and age distribution of the positive cases is shown in Table 1. A relatively high number of positive cases was found among older men, and the rate of positive cases was higher in men than in women, although the difference was not significant. Integration of the spectrum indicated that the excreted amount of AFB-Gua was in the range of 0.3 to 3 pmoles/25mL of urine (*21*). If we assume that humans excrete AFB-Gua at the same rate as rats, the range of daily exposure in the patients with positive reaction for AFB-Gua is 360 to 3600 ng/day. The lowest amount detected by HPLC and synchronous fluoroscopy corresponds to the estimated exposure levels at the lower altitude (high exposure) in Murang'a district as determined by analysis of the prepared food samples for AFB (*24*). Urine samples collected from residents in the Nairobi area served as the controls, because it is assumed that the diet here is less likely to be contaminated with AFB because of the better storage facilities for food and a higher socioeconomic level. All the positive cases from Kenyatta National Hospital lived in the rural area around Nairobi City, where it may be assumed that the food was either bought at the local open market or cultivated and stored by the patients themselves. A few of the samples showed two peaks in the synchronous fluorescent spectrum with peak emission at 415 nm and 435 nm. The chemical identity of the second product is presently unknown, but it does not represent the ring-opened form of AFB-Gua.

Table 3. Analysis of AFB-GuaI in urine from patients collected at Kenyatta National Hospital.

District	No. of samples analyzed	AFB-Gua positive cases[a]
Kiambu	39	3 (7.7)
Machakos	11	0
Meru/Embu	9	3 (33.3)
Murang'a	25	5 (20.0)
Others, including Nairobi	44	0
Total	128	11 (8.6)

[a] Limit of detectability was 0.3 pmole.

Perspective

Detection of carcinogen-DNA adducts in urine or blood samples is indirect proof for *in vivo* activation of chemical carcinogens by humans and constitutes a very specific method for monitoring exposure to carinogenic compounds. The presence of AFB-Gua in the urine samples collected in various districts of Kenya is an indication that biological activation of the carcinogen has taken place, and that the ultimate carcinogenic form of AFB has reacted with cellular nucleic acids or their precursors. We cannot rule out the possibility that AFB-Gua is from cellular RNA rather than DNA, as the adducts are identical in both nucleic acids (*24*). Furthermore, the AFB-Gua could also originate in an organ other than the liver, although this organ is considered the major site of activation of AFB in experimental animals. The presence of AFB-Gua in urine may be an indication of the presence of other potential mutagenic lesion in the liver such as the ring-opened form of AFB-Gua and apurinic sites. both lesions have been considered to be responsible for the mutagenic effect of AFB (*25,26*). However, further studies are required to establish an association between dietary intake of AFB, excretion of AFB-Gua in the urine, and the incidence of liver cancer. Before a larger epidemiological study should be initiated new analytical procedures should be developed. Application of antibodies against total AFB metabolites (*7,8*) and AFB-DNA (*27*) in immunological methods could be of great interest both for isolation and quantitation of AFB-Gua in human urine samples. Chu et al. (*28*) has produced an antiserum against aflatoxin B_{2a} that recognizes AFB-Gua and other AFB metabolites. The latter compounds will interfere with detection of AFB-Gua in the urine and an initial clean up by HPLC will be required. The sensitivity is less (50% inhibition) than in the reported method. Monoclonal antibodies against AFB-DNA have not shown any cross-reactivity with AFB-Gua using the competitive Useria assay. Donahue et al. (*8*) has taken a different approach by alkylation of the AFB-Gua isolated by HPLC with ^{3}H-dimethyl sulfate of high specific activity followed by HPLC analysis of the hydrolyzed product, 9-methylguanine. The sensitivity of this method is higher but the results are difficult to reproduce due to the many steps involved, and the method is too laborious for large epidemiological surveys. The approach taken by Sun and coworkers for identification and quantification of AFB metabolites is presently the most promising. Urine samples were concentrated by affinity chromatography using antibodies against AFB metabolites followed by HPLC analysis of the ethanol eluate (*7*). Even with improved sensitivity, the detection of AFB-Gua in urine may be an underestimation of AFB exposed individual

as AFB-Gua will only represent exposure within the last 24 to 48 hr. However, the rate of AFB-Gua-positive cases in the reported study corresponds to the number of patients with positive serology for active hepatitis infection, another major etiological agent in human liver cancer.

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